AD_____

Award Number: DAMD17-01-1-0262

TITLE: Tumor Vaccination With Cytokine-loaded Microspheres

PRINCIPAL INVESTIGATOR: Nejat K. Egilmez, Ph.D.

CONTRACTING ORGANIZATION: University of Louisville
Research Foundation, Inc.
Louisville, Kentucky 40202

REPORT DATE: September 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 074-0188*



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | September 2004 | Annual (15 Aug 2003 - 14 Aug 2004) |

**4. TITLE AND SUBTITLE**
Tumor Vaccination With Cytokine-loaded Microspheres

**5. FUNDING NUMBERS**
DAMD17-01-1-0262

**6. AUTHOR(S)**
Nejat K. Egilmez, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Louisville Research Foundation, Inc.
Louisville, Kentucky 40202

E-Mail: nejat.egilmez@louisville.edu

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. Abstract** ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

A single intra-tumoral injection of IL-12 + GM-CSF-encapsulated microspheres promoted the suppression of primary tumor growth, prevented the development of additional tumors and extended survival in the her2-neu transgenic FVB/neuN murine mammary tumor model. Induction of a long-lived, systemic, tumor-specific immune response was demonstrated in adoptive cell transfer studies, cytotoxic T-cell assays, in vivo lymphocyte subset depletion studies and analysis of serum anti-tumor antibody levels. Repeated treatment was more effective than single treatment in inducing complete tumor regression, secondary tumor suppression and enhancing survival, however long-term cures were not obtained. The failure to achieve complete cure was not due to the inability to maintain anti-tumor immunity or the infiltration of tumors by lymphocytes.

**14. SUBJECT TERMS**
immunotherapy, IL-12, GM-CSF, breast cancer, spontaneous tumors

**15. NUMBER OF PAGES**
9

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Table of Contents

## INTRODUCTION

This proposal aims to test the efficacy of a novel cytokine-based tumor vaccination strategy in the FVB/neuN murine spontaneous tumor model. Our approach involves a single intra-tumoral injection of Interleukin-12 (IL-12) and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)-loaded biodegradable microspheres to induce anti-tumor immunity. In the first year of studies the effect of this *in situ* vaccination strategy on the growth of established primary tumors, the suppression of secondary tumor development and the induction of long-term, systemic anti-tumor is investigated. The second year studies involve a more detailed analysis of the long-term anti-tumor immunity and the evaluation of this treatment strategy in a surgical model where the primary tumors are resected after vaccination. Finally, in the third year, the efficacy of combined treatment with IL-12 + GM-CSF microspheres, surgery and chemotherapy is evaluated in the surgical model.

## BODY

### Summary.

The tasks for year 1 were:

1. To monitor the FVB/neuN mice for tumor development and to characterize the model.
2. To monitor the effect of microsphere treatment on the growth of primary tumors, the development of secondary tumors and survival.
3. To evaluate the effect of microsphere dosage and vaccination schedule on the growth of primary tumors, secondary tumor development and survival.

The tasks for year 2 were:

1. To monitor the effect of IL-12 + GM-CSF microsphere treatment on the development of long-term systemic anti-tumor immunity by cytotoxic T-cell assays and by adoptive transfer of splenocytes from vaccinated mice to naïve mice.
2. To determine the efficacy of combining immunetherapy with surgery for the treatment of spontaneous tumors in a clinically relevant embodiment of the FVB/neuN model.
3. To evaluate the efficacy of repeated vaccinations with tumor cells and cytokine-encapsulated microspheres on the growth of primary tumors, secondary tumor development and survival.

The tasks for year 3 were:

1. To perform in vivo lymphocyte subset depletion experiments prior to vaccination to determine which subsets are involved in the priming of the anti-tumor immune response.
2. To treat tumor-bearing mice with chemotherapy to determine whether chemoimmunotherapy improves long-term tumor suppression.
3. Monitor post-vaccination serum cytokine levels in mice treated with chemoimmunotherapy to determine whether immune responses are enhanced after chemotherapy.
4. Monitor the ability of adoptively transferred splenocytes from mice above to suppress tumor growth in naïve mice.

All tasks for years 1 and 2 have been completed. Task 1 for year 3 has been completed. The remaining tasks for year 3 were recently initiated. The results are summarized below (Figures 1-

5).

The significant delay in the execution of the experiments proposed for year 3 is essentially due to the move of the P.I. to a new institution at the end of Year 2 (from SUNY Buffalo, NY to University of Louisville, KY). Preparation and submission of new animal protocols for P.I.'s current institute, the purchase of new animals and monitoring of these mice for tumor development, hiring and training of new personnel and finally the setting up of the new laboratory facility resulted in an 8-month break in experiments. In view of the delays caused by the move, the P.I. requested a 1 year no-cost extension to the project and this request was approved by the granting agency, extending the grant period to November 2005.

**Results.**

1. Development of spontaneous breast carcinomas in the FVB/neuN mice. See year 1 report.

2. Preparation and characterization of the IL-12 and GM-CSF-encapsulated microsphere formulations. See year 1 report.

3. Treatment of established breast tumors with IL-12 + GM-CSF microspheres and monitoring of primary tumor growth and secondary tumor development. See year 2 report.

4. Effect of microsphere dose on tumor suppression. See year 2 report.

5. Effect of treatment frequency on tumor suppression. See year 2 report.

6. Development of long-term systemic anti-tumor immunity in vaccinated mice. See year 2 report.

7. Surgical resection of treated tumors does not improve tumor recurrence rate and adversely affects secondary tumor suppression. See year 2 report.

8. Repeated vaccinations with mixtures of tumor cell suspensions and microspheres.

The original strategy involving repeated vaccinations of mice with tumor cell suspensions prepared from post-surgical tumor tissue was modified in view of the negative effects of surgical intervention on secondary tumor development (secondary tumors developed faster in post-surgical mice, second year report). Instead, we performed repeated in situ treatments of the primary tumor with IL-12 + GM-CSF microspheres without surgical intervention. Repeated in situ therapy improved primary tumor regression, suppression of secondary tumor development and overall survival (Table 1 and Figure 1). However, complete cure remained elusive.

Table 1. Repeated treatment improves primary tumor regression and suppression of secondary tumor development.

| Number of Treatments | % Mice with complete primary tumor regression | | % Secondary tumor-free mice |
|---|---|---|---|
| | Week 6 | Week 8 | Week 8 |
| 0 | 0 | 0 | 31 |
| 1 | 13 | 0 | 73 |

2 40 20 90

The above results demonstrate that treatment of mice twice (at weeks 0 and 3), in contrast to single treatment (week 0), was more effective in inducing primary tumor regression and, in preventing secondary tumor development. Tumors had regressed completely in 13% of 1x-treated and 40% of 2x-treated mice at week 6. By week 8, all regressed primary tumors recurred in 1x-treated mice. In contrast, only half of the primary tumors that had regressed recurred after 2 treatments. Moreover, the proportion of secondary tumor-free mice increased from 73% to 90% upon re-treatment. Data after week 8 is not available since some mice had to be sacrificed due to primary/secondary tumor growth starting at week 8.

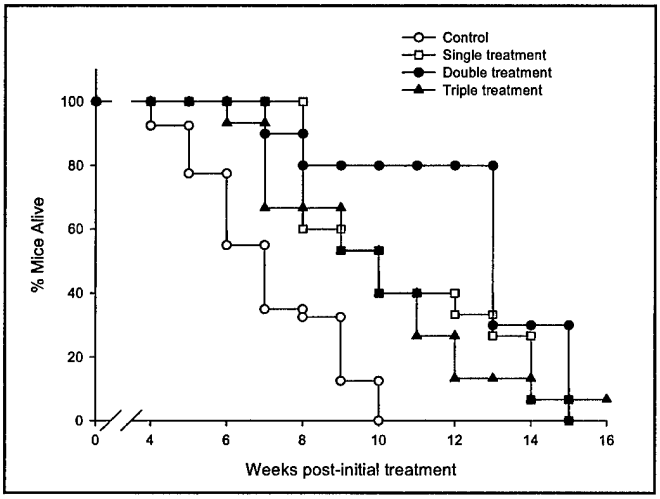


Figure 1. Long-term survival after repeated treatments. Survival was monitored after 1, 2 and 3 treatments (treatments were every 3 weeks starting at week 0). Mice were sacrificed when a tumor reached 15 mm in diameter (primary or secondary). Control mice received a single treatment of blank microspheres. Only one mouse in the triple treatment group survived tumor-free for 20 weeks. No complete cures were obtained in any of the groups. The differences between control and other groups were significant ($p \leq 0.01$, Log-Rank test), n = 16, 15, 10 and 15 for control, 1x, 2x and 3x treatments, respectively.

The results shown in Figure 3 demonstrate that although repeated treatments enhanced short-term primary tumor regression and suppression of secondary tumor development, long-term survival did not improve significantly.

9. <u>In vivo lymphocyte subset depletions demonstrate that both T- and NK cells are essential to IL-12 + GM-CSF-induced primary tumor regression</u>.

CD4+ T-cells, CD8+ T-cells and NK/NKT cells were depleted prior to the treatment of tumor-bearing mice to determine whether these lymphocyte subsets were important in mediating the anti-tumor effects of vaccination with IL-12 and GM-CSF microspheres. The results are shown in Figure 2.

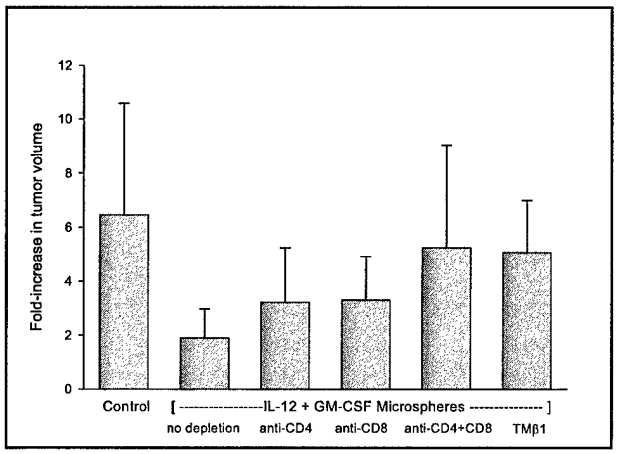


Figure 2. Both T- and NK/NKT cells are critical to primary tumor suppression. Mice were treated with anti-CD4+, anti-CD8+ (or both), or TMb1 antibody (anti-NK/NKT) starting 1 day prior to treatment (and every 4 days for a total of 5 times after that). Tumor growth was monitored for 3 weeks and fold-increase in tumor size was determined at the end of 3 weeks. The difference between control and no

depletion groups was significant ($p = 0.01$).
Differences between the no depletion
group and the CD4+CD8 depletion and the TMβ1 group were also significant ($p \leq 0.036$), n = 5 per group.

These results establish that both CD4+ and CD8+ T-cell subsets, as well as NK/NKT cells, are critical to the IL-12 + GM-CSF-induced suppression/eradication of primary tumors in the FVB/neuN model. These data also confirm our earlier studies in a transplantable tumor model where a similar anti-tumor T and NK-cell response was shown to mediate the suppression primary tumors after IL-12 + GM-CSF therapy.

10. Immunohistochemical analysis demonstrates T-cell infiltration of post-therapy primary tumors.

The above results demonstrated that T-cells were critical to tumor suppression after microsphere treatment. The kinetics of T-cell infiltration into tumors was analyzed by immunohistochemical analysis of tumor tissue sections. Representative slides are shown in Figure 3.

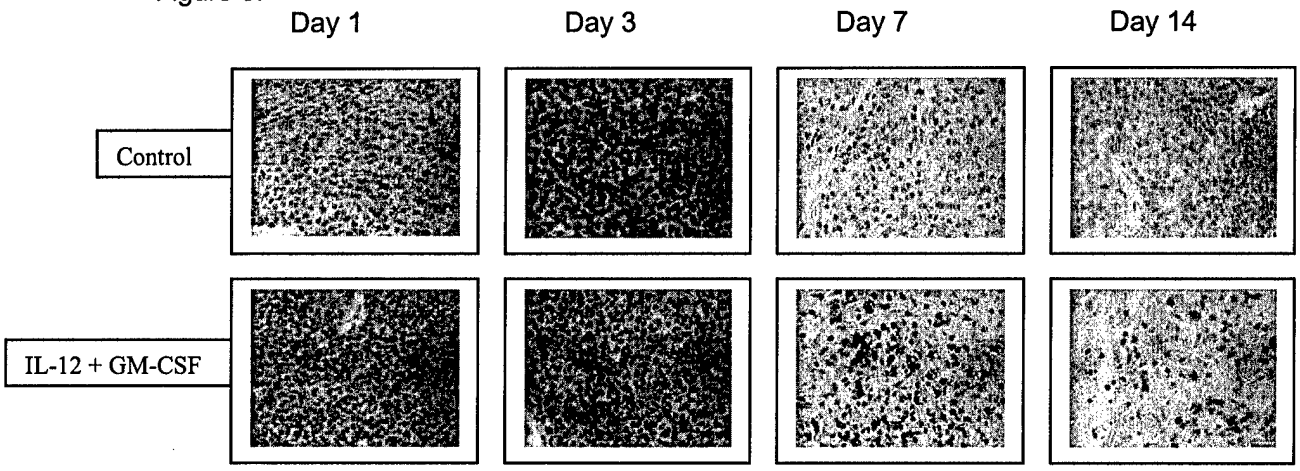


Figure 3. IL-12 + GM-CSF microsphere treatment induces T-cell infiltration into tumors. Tumors were treated with either blank (control) or IL-12 + GM-CSF microspheres. Formalin-fixed tissue sections were prepared on different days after treatment and were stained with an anti-CD3 antibody. Sections are representative of 3 tumors per group.

The above results establish that our therapy induces a potent anti-tumor T-cell response that infiltrates tumors effectively. However, the infiltration kinetics indicate that the anti-tumor T-cell response peaks at one week after therapy and gradually diminishes after that despite tumor persistence (analysis of tumor sections 3-7 weeks after treatment indicated sporadic low-level infiltration, data not shown).

11. Analysis of serum antibody levels demonstrates the development of anti-tumor antibodies in mice treated with IL-12 + GM-CSF microspheres.

All previous studies focused on the development of an anti-tumor T-cell response. Both IL-12 and GM-CSF have also been shown to enhance the development of potent antibody responses. In an attempt to dissect the anti-tumor immune mechanisms that are induced by our

strategy further, the development of anti-tumor antibodies in the sera of treated mice was investigated. The results are shown in Figure 4.

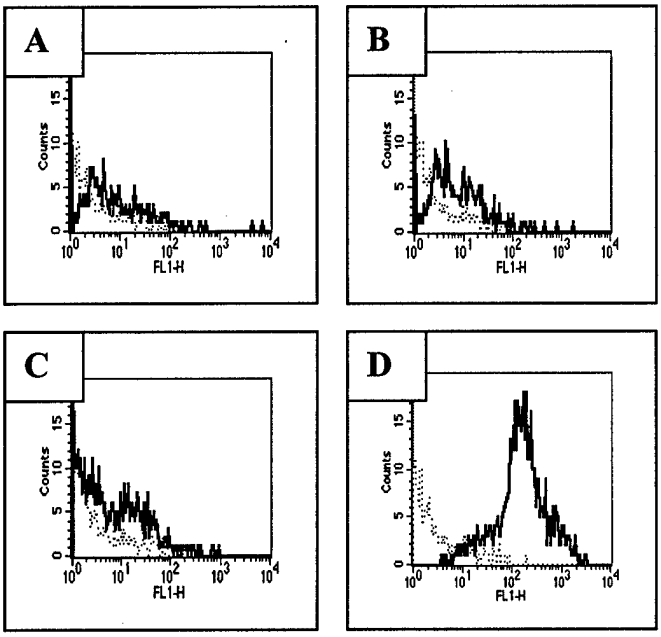


Figure 4. Development anti-tumor antibodies after treatment of mice with IL-12 + GM-CSF-encapsulated microspheres. Mice bearing spontaneous established tumors (100-200 $mm^3$) were treated either with blank microspheres (A) or IL-12 + GM-CSF microspheres (B-D). Sera were collected on day 18 post-treatment (A and B). Booster treatments were performed with IL-12 + GM-CSF microspheres on days 21 and 42, and sera were collected on days 28 (C) and 49 (D). The presence of anti-tumor antibodies in the sera was determined by flow cytometry analysis of serum antibody binding to tumor cell suspensions. Dotted line = control IgG, solid line = serum antibody. Serum samples were pooled from 3 mice in each group.

These data demonstrate that our strategy induces the development of an anti-tumor B-cell response as well as T-cell activation. However, the development of significant levels of anti-tumor antibodies requires several booster treatments with IL-12 + GM-CSF microspheres.

12. Chemoimmunotherapy studies.

We are currently performing dose-response studies to determine the effect of increasing doses of chemotherapeutic agents on serum leukocyte levels. Once these studies are completed, chemoimmunotherapy experiments will be initiated.

## KEY RESEARCH ACCOMPLISHMENTS

- Chronic therapy resulted in superior anti-tumor immunity, however this did not result in a significant improvement to long-term survival.
- Primary tumor suppression/eradication was shown to be mediated by both T- and NK/NKT cells.
- Analysis of T-cell infiltration kinetics into the tumors demonstrated that the T-cells successfully infiltrated tumors after treatment however the anti-tumor T-cell response peaked at day 7 post-treatment and gradually diminished afterwards resulting in tumor recurrence.
- The development of a potent anti-tumor B-cell response was demonstrated in treated mice. However, this response required multiple treatments to reach significant levels.

## REPORTABLE OUTCOMES

A manuscript summarizing the results obtained in the first 2 years of these studies is currently in preparation and will be submitted to Journal of Immunology.

Abstracts for poster presentation have been submitted to the International Society of Biological Therapy Meeting in San Fransisco, CA (November 2004) and the Keystone Meeting on Basic Aspects of Tumor Immunology in Keystone, CO (March 2005). The abstract submitted to the iSBTC meeting was chosen for oral presentation.

## CONCLUSIONS

The results reported here demonstrate that treatment with IL-12 + GM-CSF microspheres induces both innate (NK-cell) and adaptive (T- and B-cell) anti-tumor responses which successfully infiltrate and suppress tumors. However the anti-tumor immunity (primarily the T-cell response) peaks by day 7 post-therapy and then diminishes resulting in transient tumor suppression. Repeated treatments improve tumor suppression, however are unable to achieve complete long-term cure. In Aim 3 studies we will investigate whether combining low-dose chemotherapy with chronic immune-therapy can achieve superior long-term tumor suppression in the FVB/neuN model. These data have important clinical implications since they demonstrate that a therapeutic strategy which works well in traditional transplantable models is not as effective in the spontaneous disease setting.